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$G\alpha_{12}$ and $G\alpha_{13}$ Are Phosphorylated during Platelet Activation*

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The ubiquitously expressed G-proteins G_{12} and G_{13} whose function is currently not clear have been shown to be activated in platelet membranes through receptors that stimulate platelet aggregation. We used intact human platelets to determine whether α subunits of both G-proteins can be phosphorylated under physiological conditions. Activation of human platelets by thrombin and the thromboxane A_2 receptor agonist U46619 lead to phosphorylation of $G\alpha_{12}$ and $G\alpha_{13}$. Phosphorylation occurred rapidly after addition of thrombin and was not mediated by glycoprotein IIb/IIIa (integrin $\alpha_{IIb}\beta_3$) activation. Phosphorylation of $G\alpha_{12}$ and $G\alpha_{13}$ could be mimicked by phorbol 12-myristate 13-acetate, and thrombin-induced phosphorylation was inhibited by the protein kinase C inhibitor calphostin C indicating an involvement of protein kinase C in $G\alpha_{12/13}$ phosphorylation induced by thrombin in human platelets. The phosphorylation of both G protein α subunits was reconstituted in COS-7 cells cotransfected with $G\alpha_{12}$ or $G\alpha_{13}$ and different protein kinase C isoforms. Among the protein knase C isoforms tested, protein kinase C β, δ, and ϵ were most effective in promoting phosphorylation of $G\alpha_{12}$ and $G\alpha_{13}$ in a phorbol 12-myristate 13-acetate-dependent manner. These data demonstrate that $G\alpha_{12}$ and $G\alpha_{13}$ are phosphorylated under *in vivo* conditions and that this phosphorylation involves protein kinase C.

G-proteins[1] are heterotrimeric proteins which functionally couple a variety of receptors to effectors that include adenylyl cyclase, phospholipase C-β, cyclic GMP phosphodiesterase, and various ion channels (1–4). Heterotrimeric G-proteins consist of an α-subunit which binds and hydrolyzes GTP, a β- and a γ-subunit. The identity of the G-protein α-subunit defines the heterotrimeric G-protein. Based on sequence and functional homologies the α-subunits can be divided into four families ($G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_q$, and $G\alpha_{12}$) (5). In a given cell, several signal transduction pathways involving different heterotrimeric G-proteins operate in parallel, and there is increasing evidence that the G-protein mediated signal transduction system undergoes adaptation and cross-regulation at many different levels. Protein phosphorylation and dephosphorylation is an important regulatory mechanism found in a variety of signal transduction systems. Several reports have shown that the α-subunits of some members of the $G_{i/o}$ family can be phosphorylated *in vivo* and *in vitro*. In platelets, thrombin, thromboxane A_2 analogs, and phorbol esters lead to a protein kinase C mediated phosphorylation of $G\alpha_z$ (6, 7). Furthermore, this phosphorylation blocks interaction of $G\alpha_z$ with $G\beta\gamma$ (8). In addition, several reports have shown that $G\alpha_i$ can be phosphorylated in various systems under conditions leading to protein kinase C and cGMP dependent protein kinase activation (9–12). Most of these reports suggest that $G\alpha_i$ phosphorylation correlates with inactivation of signaling via this G-protein (9, 11, 12).

The G-proteins G_{12} and G_{13} are ubiquitously expressed and together constitute the G_{12} family (13). Studies using constitutively activated forms of both α-subunits have provided evidence that G_{12} and G_{13} are involved in the regulation of different downstream effectors and signaling pathways, *e.g.* the Na^+/H^+exchanger (14–16), the Jun-kinase/stress-activated protein kinase pathway (17) and the Rho-dependent formation of stress fibers (18). However, the effectors directly regulated by G_{12} and G_{13} are currently unknown. It is clear that both G-proteins are distinct from most other G-proteins with regard to the biochemical properties of their α-subunits. The receptor-catalyzed guanine nucleotide exchange rate of G_{12} and G_{13} is very low when measured in membrane fractions (19), and purified $G\alpha_{12}$ and $G\alpha_{13}$ exhibit very slow rates of GDP dissociation and GTP association (20, 21).

Platelets are a well described model system for studies of G-protein mediated signal transduction processes. In human platelet membranes both, $G\alpha_{12}$ and $G\alpha_{13}$, have been shown to be activated through thrombin and thromboxane A_2 receptors (19). We therefore chose intact human platelets to test whether $G\alpha_{12}$ and $G\alpha_{13}$ can be phosphorylated under *in vivo* conditions. Our data show that treatment of human platelets with physiological platelet activators leads to a rapid and sustained phosphorylation of both G-protein α-subunits and that this phosphorylation is mediated by protein kinase C.

EXPERIMENTAL PROCEDURES

Materials—Thrombin, thrombin receptor peptide (SFLLRNPNDKYEPF), U46619, phorbol 12-myristate 13-acetate (PMA), protein A-Sepharose, and Arg-Gly-Asp-Ser (RGDS) were from Sigma. Calphostin C was from Calbiochem (San Diego, CA) and [^{32}P]orthophosphate was from DuPont NEN. Anti-$G\alpha_{12}$ and -$G\alpha_{13}$ antisera were raised against peptides representing the carboxyl-terminal 10 amino acids of $G\alpha_{12}$ and $G\alpha_{13}$, respectively. The cDNAs of $G\alpha_{12}$ and $G\alpha_{13}$ were carried by the cytomegalovirus promotor containing vector pCIS. A β-galactosidase construct inserted into pCIS was used as a transfection control. The PKCα, βI, δ, ϵ, and ζ cDNAs were in the expression vector pMT2, and the cDNA of PKCη was carried by pKSI (22).

Isolation of Platelets—Venous blood was collected from healthy drug-free volunteers and anticoagulated with 6 mM citric acid, 12 mM sodium citrate. Platelet isolation was carried out at room temperature. Platelet rich plasma was prepared by centrifugation of whole blood for 20 min at 200 × *g*. Platelet rich plasma was adjusted with citrate buffer to pH 6.5 and centrifuged two times at 200 × *g* for 15 min to remove contaminating cells. Thereafter, platelets were pelleted by centrifugation for 10 min at 700 × *g* and washed once in buffer A containing 138 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 5 mM $NaHCO_3$, 5.5 mM glucose, 0.5 mM EDTA, 0.1% (w/v) bovine serum albumin, 0.5 unit/ml apyrase, and 20 mM

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[1] The abbreviations used are: G-protein, heterotrimeric guanine nucleotide-binding protein; U46619, 11α,9α-epoxymethano-prostaglandin H_2; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium.

Hepes (pH 7.4). Platelets were metabolically labeled by incubation in the above buffer containing 1 mCi/ml [^{32}P]orthophosphate for 2 h at room temperature.

Immunoprecipitation—Metabolically labeled platelets were washed once in buffer A (see above) and about 2×10^9 platelets were preincubated for 3 min at 37 °C in 200 μl of the same buffer devoid of apyrase and containing 1 mM $CaCl_2$. Incubation was initiated by addition of indicated agents (10 μl) and was stopped by adding 50 μl of 5% (w/v) sodium dodecyl sulfate, 20 mM EDTA, 50 mM NaF, 50 mM $Na_4P_2O_7$. After 10 min at room temperature, 1 ml of precipitating buffer (1% (v/v) Nonidet P-40, 1% (w/v) desoxycholate, 0.5% (w/v) sodium dodecyl sulfate, 150 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 10 mM NaF, 5 mM $Na_4P_2O_7$, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 mM Tris·HCl (pH 7.4)) was added, and lysates were centrifuged for 10 min at 12000 $\times$ *g*. Thereafter, supernatants were precleared by incubation with 50 μl of 12.5% (w/v) protein A-Sepharose beads for 1 h at 4 °C at constant rotation. Sepharose beads were pelleted and supernatants were incubated for another 1–1.5 h in the presence of 10 μl antiserum. After addition of 50 μl of 12.5% (w/v) protein A-Sepharose beads samples were incubated for 1 h at 4 °C, and immunocomplexes were washed and prepared for SDS-PAGE as described (19).

Expression and Phosphorylation of $G\alpha_{12}$ and $G\alpha_{13}$ in COS-7 Cells—COS-7 cells were cultured as described (23). For transfection experiments, cells were seeded in 12-well plates at a density of 1×10^5 cells per well and grown overnight. Cells were then washed with phosphate-buffered saline solution and 1 μg of plasmid DNA mixed with 5 μl of LipofectAMINE (Life Technologies, Inc.) in 0.5 ml of Opti-MEM was added to each well. In control experiments, the total amount of DNA was maintained constant by adding DNA from a vector encoding β-galactosidase. After 5 h, 0.5 ml of DMEM containing 20% (v/v) fetal bovine serum was added, and cells were used for experiments about 45 h later. For metabolic labeling, cells were washed 3 times with DMEM devoid of phosphate. Thereafter cells were incubated for 2 h in 0.4 ml phosphate-free DMEM containing 0.5 mCi/ml [^{32}P]orthophosphate and 20 mM Hepes·NaOH (pH 7.4). Cells were then washed once with phosphate-free DMEM and incubated in the absence or presence of the indicated agents at 37 °C. Reactions were stopped by removing incubation medium and adding of 100 μl of 1% (w/v) sodium dodecyl sulfate, 5 mM EDTA, 20 mM NaF, 10 mM $Na_4P_2O_7$. Immunoprecipitation of proteins was done as described above.

Determination of Protein Kinase C Activity—Transfected COS-7 cells were washed twice with phosphate-buffered saline. Phosphorylation was initiated by adding 0.5 ml of permeabilization solution consisting of 115 mM KCl, 15 mM NaCl, 0.5 mM $MgCl_2$, 20 mM Hepes·NaOH (pH 7), 1 mM EGTA, 100 μM [γ-^{32}P]ATP (100 mCi/mmol), 50 μM each of PKC substrate peptides A and B, 20 μM digitonin, and 0.72 mM $CaCl_2$ (to give a free Ca^{2+} concentration of 500 nM) (24). Cells were incubated in the absence or presence of 100 nM PMA for 10 min at 37 °C. 400 μl of the supernatant were then spotted onto phosphocellulose paper (Whatman P81) and dried. Filter papers were washed five times in 30% (v/v) acetic acid and counted. PKC substrate peptides A and B represent the pseudosubstrate sites of PKCζ (IYRRGSRRLRKL) and PKCδ (MNRRGSIKQAKI) except for the substitution of serine in place of alanine.

RESULTS

Human platelets metabolically labeled with [^{32}P]orthophosphate were incubated with different agents which lead to the inhibition or activation of platelet function, platelet lysates were prepared, and $G\alpha_{12}$ and $G\alpha_{13}$ were then immunoprecipitated from the lysates with specific antisera. No radioactive phosphate was found in $G\alpha_{12}$ and $G\alpha_{13}$ immunoprecipitated from untreated platelets or platelets incubated in the presence of the PGI_2 receptor agonist cicaprost, forskolin, or sodium nitroprusside (data not shown). However, treatment of platelets with the platelet activators thrombin and thromboxane A_2 analogue U46619 as well as with the phorbol ester PMA resulted in a marked phosphorylation of $G\alpha_{12}$ and $G\alpha_{13}$ (Fig. 1). Thombin receptor peptide mimicked the effect of thrombin, demonstrating the specific activation of the G-protein coupled thrombin receptor. The extent of phosphorylation induced by U46619 and thrombin receptor peptide was lower than that promoted by PMA and thrombin. To confirm the specificity of the antisera used for immunoprecipitation, the anti-$G\alpha_{12}$ and anti-$G\alpha_{13}$ antisera were preincubated with the individual peptides against which they were raised. Fig. 2 shows that immunoprecipitation of phosphorylated 42-kDa proteins by anti-$G\alpha_{12}$ and anti-$G\alpha_{13}$ antisera was blocked by the respective peptides. There was no cross-reactivity between both antisera as the $G\alpha_{12}$ peptide was unable to block precipitation by the $G\alpha_{13}$ antiserum and the $G\alpha_{13}$ peptide had no effect on the precipitation by the $G\alpha_{12}$ antiserum. No phosphorylated protein was precipitated by preimmune sera (Fig. 2). Similarly and in agreement with previous studies (7), immunoprecipitation with an antiserum recognizing the C terminus of $G\alpha_{q/11}$ did not precipitate any phosphorylated $G\alpha_{q/11}$ from lysates of platelets treated with different agents (data not shown).

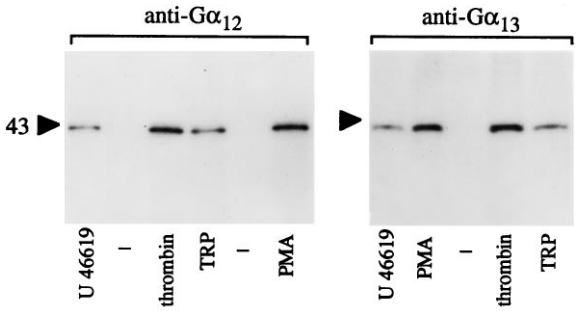

FIG. 1. **Phosphorylation of $G\alpha_{12}$ and $G\alpha_{13}$ by different stimuli in human platelets.** Platelets were metabolically labeled with [^{32}P]H_3PO_4 and were subsequently incubated in the absence or presence of 3 μM U46619, 5 units/ml thrombin, 100 μM thrombin receptor peptide (*TRP*) and 1 μM PMA at 37 °C for 3 min as described under "Experimental Procedures." Following solubilization of the platelets, precipitates were prepared with an anti-$G\alpha_{12}$ (*left panel*) and with an anti-$G\alpha_{13}$ antiserum (*right panel*). Shown are autoradiograms of SDS-polyacrylamide gels which are representative of three experiments conducted under identical conditions. The position of the 43-kDa standard protein is shown on the left of each panel.

Time course experiments revealed a rapid and sustained phosphorylation of $G\alpha_{12}$ and $G\alpha_{13}$ in response to thrombin (Fig. 3). Phosphorylation of both proteins was maximal 10 s after addition of thrombin, whereas phosphorylation in response to PMA was maximal not earlier than 2 min after PMA was added. To rule out that phosphorylation of both proteins was dependent on activation of glycoprotein IIb/IIIa (integrin $\alpha_{IIb}\beta_3$) we incubated platelets with the peptide RGDS prior to addition of thrombin (Fig. 4). Pretreatment of platelets with RGDS, an inhibitor of $\alpha_{IIb}\beta_3$-fibrinogen binding, had no effect on the thrombin induced phosphorylation of $G\alpha_{12}$ and $G\alpha_{13}$, whereas it prevented formation of a platelet aggregate which was usually visible about 3–4 min after addition of thrombin. Thus, thrombin induced $G\alpha_{12}$ and $G\alpha_{13}$ phosphorylation is an event occurring early during platelet activation.

Since the effect of thrombin was mimicked by the protein kinase C activating phorbol ester PMA and since platelet activation is known to involve activation of protein kinase C (25–27), we investigated the role of PKC in $G\alpha_{12}$ and $G\alpha_{13}$ phosphorylation. Fig. 4 shows that the effects of thrombin and PMA, both employed at maximally effective concentrations were not additive, suggesting that both agents acted through at least similar mechanisms involving the phosphorylation of the same residues. Calphostin C, a potent inhibitor of diacylglycerol and Ca^{2+}-dependent isoforms of protein kinase C (28) inhibited the effect of thrombin on the phosphorylation of $G\alpha_{12}$ and $G\alpha_{13}$ in a concentration dependent manner in human platelets (Fig. 5), indicating that protein kinase C is involved in the thrombin dependent phosphorylation of $G\alpha_{12}$ and $G\alpha_{13}$.

To further elucidate the mechanism of $G\alpha_{12}/G\alpha_{13}$ phosphorylation and to study the possible involvement of specific protein kinase C isoforms, we tried to reconstitute the protein kinase C dependent phosphorylation of both G-protein α-sub-

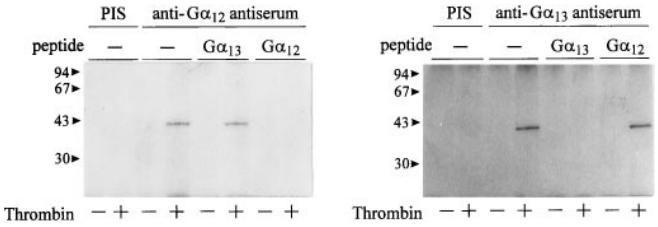

FIG. 2. **Specificity of antisera used for precipitation of $G\alpha_{12}$ and $G\alpha_{13}$ from human platelet lysates.** Metabolically labeled platelets were incubated in the absence (*thrombin* −) or presence of 5 units/ml thrombin (*thrombin* +) for 2 min. Platelets were solubilized, and the indicated antisera were added (*PIS*, preimmune serum). Prior to addition, the anti-$G\alpha_{12}$ and the anti-$G\alpha_{13}$ antiserum had been preincubated overnight at 4 °C in the absence (*peptide* −) or presence of 10 μg/ml of the indicated C-terminal decapeptides of $G\alpha_{12}$ and $G\alpha_{13}$ against which the antisera had been raised. Immunoprecipitation was performed as described under "Experimental Procedures," and precipitated proteins were subjected to SDS-PAGE. Shown are autoradiograms of SDS-polyacrylamide gels with the position of standard proteins indicated on the left. These results represent one of two experiments.

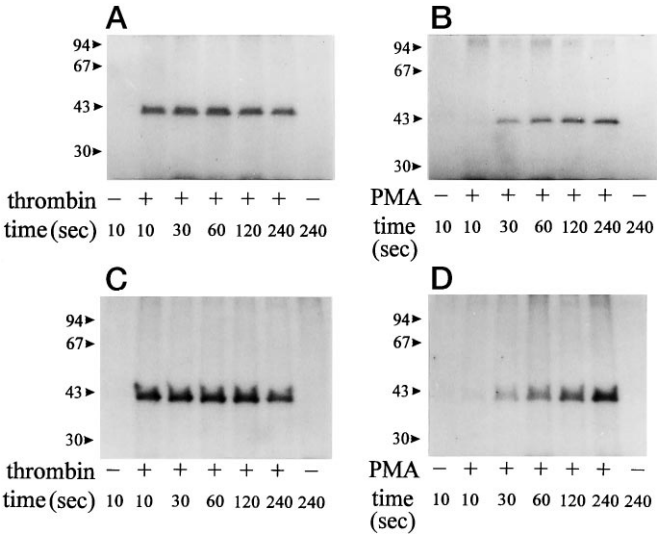

FIG. 3. **Time courses of thrombin and PMA induced phosphorylation of $G\alpha_{12}$ and $G\alpha_{13}$ in human platelet.** Labeled platelets were incubated in the absence or presence of 5 units/ml thrombin (*thrombin* −/+, *panels A* and *C*) and 1 μM PMA (*PMA* −/+, *panels B* and *D*) for the indicated time periods, and platelets were solubilized. Proteins were immunoprecipitated with the anti-$G\alpha_{12}$ (panels A and B) or the anti-$G\alpha_{13}$ antiserum (*panels C* and *D*) as described. Precipitated proteins were subjected to SDS-PAGE. Shown are autoradiograms of SDS-polyacrylamide gels with the position of standard proteins indicated on the left (kDa). These results represent one of two experiments.

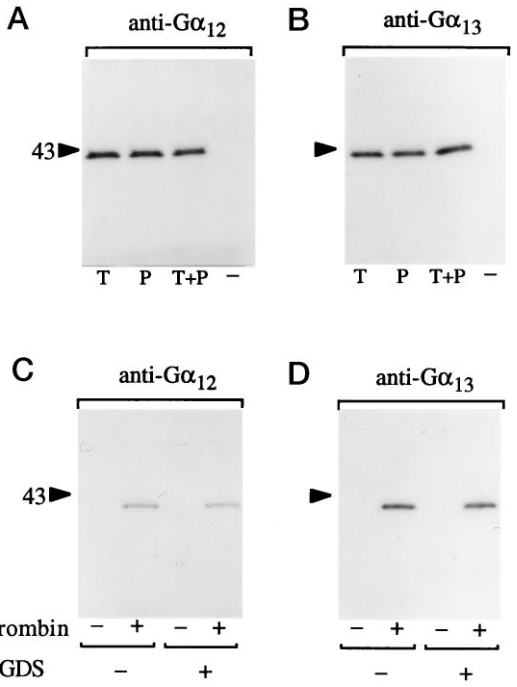

FIG. 4. **Lack of additivity of thrombin and PMA stimulated $G\alpha_{12}/G\alpha_{13}$ phosphorylation and effect of RGDS on thrombin dependent phosphorylation of $G\alpha_{12}$ and $G\alpha_{13}$ in human platelet.** *Panels A and B*, metabolically labeled platelets were incubated without (−) or with 10 units/ml thrombin (*T*), 1 μM PMA (*P*) and 10 units/ml thrombin + 1 μM PMA (*T+P*) for 3 min. *Panels C* and *D*, platelets were preincubated without or with 0.5 mM RGDS (*RGDS* −/+) for 5 min. Thereafter, 5 units/ml thrombin (*thrombin* +) or vehicle (*thrombin* −) was added and platelets were incubated for 2 min at 37 °C. Solubilized platelets were then incubated with the anti-$G\alpha_{12}$ (*panels A* and *C*) or the anti-$G\alpha_{13}$ antiserum (*panels B* and *D*) as described. Precipitated proteins were subjected to SDS-PAGE. Shown are autoradiograms of SDS-polyacrylamide gels with the position of the 43-kDa standard protein shown on the left.

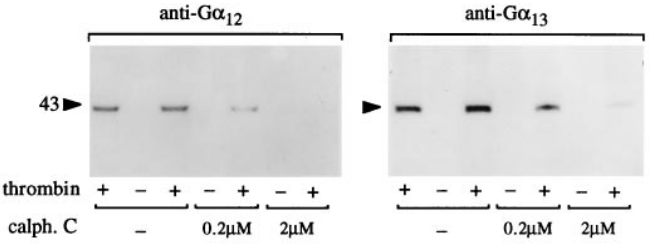

FIG. 5. **Effect of calphostin C on thrombin induced phosphorylation of $G\alpha_{12}$ and $G\alpha_{13}$ in human platelet.** [^{32}P]H_3PO_4-labeled platelets were preincubated without or with the indicated concentration of calphostin C (*calph. C*) for 10 min. Thereafter, 5 units/ml thrombin (*thrombin* +) or vehicle (*thrombin* −) was added, and platelets were incubated for 2 min at 37 °C. Solubilized platelets were then incubated with the anti-$G\alpha_{12}$ (*left panel*) or the anti-$G\alpha_{13}$ antiserum (*right panel*) as described. Precipitated proteins were subjected to SDS-PAGE. Shown are autoradiograms of SDS-polyacrylamide gels with the position of the 43-kDa standard protein shown on the left. Samples loaded on the left lane of each panel contained 0.2% (v/v) Me_2SO during preincubation and incubation with thrombin in order to rule out inhibitory effects of Me_2SO, which was present at this concentration in samples preincubated with 2 μM calphostin C.

units in COS-7 cells. Fig. 6 shows that expression of different protein kinase C isoforms in COS-7 cells resulted in increased basal protein kinase C activity measured in permeabilized cells using PKC substrate peptides. Incubation of cells with the phorbol ester PMA increased the protein kinase C activity in cells expressing the protein kinase C isoforms α, β, δ, ϵ, and η, whereas the kinase activity in cells transfected with the atypical PKC isoform ζ was unresponsive to PMA. These data show that protein kinase C isoforms expressed in COS-7 cells were functionally active. We then coexpressed individual PKC isoforms and $G\alpha_{12}$ or $G\alpha_{13}$ and measured the phosphorylation of both G-protein α-subunits in response to PMA (Fig. 7). In metabolically labeled COS-7 cells transfected only with a control plasmid encoding β-galactosidase and incubated in the presence of PMA, no phosphorylated $G\alpha_{12}$ or $G\alpha_{13}$ was immunoprecipitated, whereas in cells transfected with $G\alpha_{12}$ or $G\alpha_{13}$, PMA induced the phosphorylation of both proteins presumably through the activation of endogenous protein kinase C. This PMA-induced phosphorylation of $G\alpha_{12}$ as well as of $G\alpha_{13}$ was unchanged in cells coexpressing the PKC isoforms α and η, indicating that these PKC isoforms were unable to mediate the effect of PMA on phosphorylation of $G\alpha_{12}$ and $G\alpha_{13}$ in our cotransfection system. However, in cells cotransfected with PKCβ, δ, or ϵ and $G\alpha_{12}$ or $G\alpha_{13}$ PMA-induced phosphorylation of each G-protein α-subunit was increased compared to cells

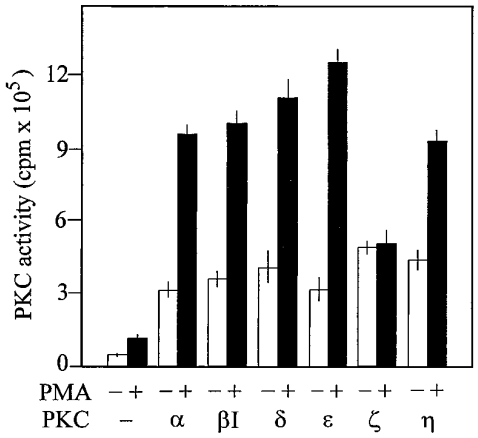

FIG. 6. **Basal and PMA stimulated activity of different PKC isoforms expressed in COS-7 cells.** COS-7 cells were transfected with cDNAs encoding the indicated isoforms of PKC. After 45 h, cells were then incubated in the absence or presence of 100 nM PMA, and PKC activity was determined as described under "Experimental Procedures." Shown are mean values ± S.D.

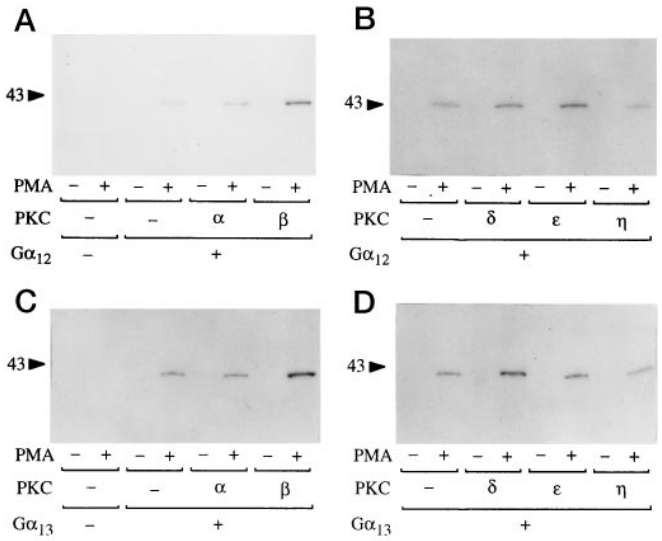

FIG. 7. **PMA induced phosphorylation of $G\alpha_{12}$ and $G\alpha_{13}$ in COS-7 cells expressing different PKC isoforms and $G\alpha_{12}$ or $G\alpha_{13}$.** COS-7 cells were cotransfected with cDNAs encoding the indicated isoforms of PKC and cDNAs encoding $G\alpha_{12}$ (*panels A* and *B*) or $G\alpha_{13}$ (*panel C* and *D*). The total amount of DNA was maintained constant by adding DNA of a vector encoding β-galactosidase. Cells were then incubated in the absence or presence of 500 nM PMA (*PMA* −/+) for 5 min, and solubilized cells were incubated with the anti-$G\alpha_{12}$ (*panels A* and *B*) or the anti-$G\alpha_{13}$ antiserum (*panels C* and *D*) as described. Precipitated proteins were subjected to SDS-PAGE. Shown are autoradiograms of SDS-polyacrylamide gels with the position of the 43-kDa standard protein shown on the left.

transfected only with the Gα-subunit. The amount of $G\alpha_{12}$ and $G\alpha_{13}$ expressed in different cotransfected cells was the same when tested by Western blotting of cell lysates (data not shown). Quantification of radioactivity incorporated into $G\alpha_{12}$ and $G\alpha_{13}$ by using the PhosphorImager showed that PMA stimulated phosphorylation about 4.5-fold in cells expressing PKCβ compared to control cells, whereas PMA stimulated phosphorylation of $G\alpha_{12}$ and $G\alpha_{13}$ in cells cotransfected with PKCδ and ε was slightly lower (2–3.5-fold). These data demonstrate that the PKC-dependent phosphorylation of $G\alpha_{12}$ and $G\alpha_{13}$ observed in human platelets can be reconstituted by cotransfection of different PKC isoforms and both G-protein α-subunits in COS-7 cells.

DISCUSSION

The object of this study was to test whether members of the $G\alpha_{12}$ family can be phosphorylated under physiological conditions. Human platelets were used as an experimental system since they contain both $G\alpha_{12}$ and $G\alpha_{13}$ as well as a variety of well described signaling pathways. We demonstrate that in human platelets both $G\alpha_{12}$ and $G\alpha_{13}$ undergo phosphorylation in response to the platelet activators thrombin and thromboxane A_2. Phosphorylation occurred independent of glycoprotein IIb/IIIa (integrin $\alpha_{IIb}\beta_3$) activation and was maximal within seconds after addition of thrombin to platelets, indicating that it is an event occurring during the first phase of platelet activation. Phosphorylation of $G\alpha_{12}$ and $G\alpha_{13}$ could also be observed after addition of phorbol ester PMA, and thrombin induced phosphorylation was blocked by the protein kinase C inhibitor calphostin C, strongly suggesting that phosphorylation of both G-proteins involves protein kinase C. PKC activation can be observed very early after activation of platelets by thrombin and is thought to play an important role in agonist stimulated activation of platelets (25–27). Several PKC isoforms have been described in platelets including isoforms α, β, δ, and ζ (25–27, 29, 30). It was demonstrated that thrombin induces a very rapid rise in diacylglycerol levels followed by a sustained translocation of PKCα, β, and ζ from the cytosolic to the membrane fraction of platelets (24). Both the thrombin induced diacylglycerol generation as well as the translocation of PKC isoforms were independent of glycoprotein IIb/IIIa activation (24). Thus, this rapid activation of PKC can well account for the phosphorylation of $G\alpha_{12}$ and $G\alpha_{13}$ in platelets. The PKC isoform β is a good candidate for mediating the phosphorylation of $G\alpha_{12}$ and $G\alpha_{13}$ as seen in COS-7 cells cotransfected with PKCβ and $G\alpha_{12}$ or $G\alpha_{13}$, addition of PMA led to a very efficient phosphorylation of both G-proteins compared to control cells. It is, however, not clear whether PKC directly phosphorylates $G\alpha_{12}$ and $G\alpha_{13}$ or whether phosphorylation occurs through another yet unknown process involving PKC.

$G\alpha_{12/13}$ phosphorylation in activated platelets bears some resemblance to the phosphorylation of $G\alpha_z$ in platelets (6, 7). $G\alpha_z$ was shown to be the substrate of PKC and the primary site of phosphorylation has been mapped to Ser^{27} of $G\alpha_z$ whereas Ser^{16} serves as a secondary site for PKC phosphorylation. Ser^{27} and Ser^{16} are situated in the N-terminal region of the α-subunit which makes contact with Gβγ (31), and biochemical evidence has been provided that phosphorylation of $G\alpha_z$ by PKC blocks interaction with Gβγ (8). However, the physiological role of $G\alpha_z$ and its phosphorylation remains obscure. In analogy to $G\alpha_z$ we mutagenized Ser^{31} of $G\alpha_{13}$ which corresponds to Ser^{16} in $G\alpha_z$ as well as Ser^{39} of $G\alpha_{13}$ which also lies in the domain which is thought to contact Gβγ. Both the single mutants as well as the double mutant were, however, still phosphorylated in response to phorbol esters when expressed in COS-7 cells (data not shown), indicating that phosphorylation of $G\alpha_{13}$ occurs at different residues and that the functional consequences of $G\alpha_{13}$ phosphorylation are probably different from those of $G\alpha_z$ phosphorylation.

G_{12} and G_{13} have been shown to be activated via thrombin and thromboxane A_2 receptors in human platelet membranes (19), suggesting that both G-proteins are involved in the activation of platelets. Interestingly, both receptors as shown in the present study induce phosphorylation of both G-protein α-subunits in intact human platelets. Both receptors also activate G_q family members leading to the activation of PLCβ and the subsequent rise in $[Ca^{2+}]_i$ and PKC activation. Thus, it is possible that thrombin and thromboxane A_2 induced $G\alpha_{12/13}$

phosphorylation in platelets results from activation of PLCβ by $G\alpha_q$ representing "cross-talk" between the G_q/PLCβ pathway and $G_{12/13}$. Constitutively active mutants of $G\alpha_{12}$ and $G\alpha_{13}$ were recently shown to stimulate stress fiber formation and focal adhesion assembly through the small guanine nucleotide binding protein Rho in Swiss 3T3 cells, whereas activated forms of $G\alpha_q$ or $G\alpha_{i2}$ had no effect (18). Aggregation of platelets in response to thrombin has been found to be inhibited by *Clostridium botulinum* C3 exoenzym, which ADP-ribosylated and inhibits Rho (32). Thus, mediating the activation of Rho in platelets exposed to thrombin is a possible function of $G\alpha_{12}$ and $G\alpha_{13}$. A striking feature of $G\alpha_{12}$ and $G\alpha_{13}$ studied in purified form is their low rate of guanine nucleotide turnover (20, 21). Similarly, both α-subunits showed a very slow rate of GTP binding in platelet membranes; GTP binding stimulated through the thromboxane A_2 and thrombin receptor reached a maximum only after 30 min (19). This guanine nucleotide exchange rate seems to be far to slow to allow $G\alpha_{12}$ and $G\alpha_{13}$ to play a significant role during platelet activation which takes place within a few minutes. There might be mechanisms leading to a faster activation of G_{12} and G_{13} which eluded experimental analysis in cell free systems. It is tempting to speculate that the rapid phosphorylation of $G\alpha_{12}$ and $G\alpha_{13}$ we observed in activated platelets may be part of a mechanism which leads to a faster activation of $G_{12/13}$ and triggers or sustains their function in platelet activation.

Taken together, we provide evidence that $G\alpha_{12}$ and $G\alpha_{13}$ are rapidly phosphorylated in activated platelets in a PKC-dependent manner and that this *in vivo* phosphorylation can be reconstituted in a cotransfected system. Once, the effector function directly regulated by G-proteins of the $G\alpha_{12}$ family has been characterized, the effect of PKC-mediated phosphorylation on $G_{12/13}$ signaling can be determined.

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Note Added in Proof—Recently, Kozasa and Gilman (33) showed that purified $G\alpha_{12}$ can be phosphorylated by PKCα *in vitro*.

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